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14. ABSTRACT
(1) MCF7 cells are more resistant to SAM50 and TOM40 knockdown. This could be due to their ability to activate the transcription machinery to compensate for the loss due siRNA. (2) All the cell lines try to compensate for the loss of mitochondrial beta barrel proteins by up regulating the respiratory enzyme COXIV. (#) While suppression of SAM50, TOM40, and VDAC1 could result in suppression of epithelial-mesenchymal transition in MCF7, it would prove counter-productive in MDA468, where the expression of Snail was enhanced after knockdown. (4) Since TOM40 is the main channel of entry of nuclear encoded proteins, its knockdown should result in suppresseion of mitochondrial activity. The results of western blot suggest contrary to this in some cases. This could be a result of negative feedback loops originating from mitochondria, but this hypothesis needs to be investigated in detail.(5) The variation in the level of vimentin between different cell lines suggest that while progression of some metastatic adenocacinomas like those similar to MCF7 type may be treated with mitochondrial protein knockdown, this will result in promoting aggressiveness in other categories of breast tumors.

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INTRODUCTION

Mitochondria play a crucial role in carcinogenesis via enhanced metabolic turnover. Additionally mitochondria serve as a hexokinase activation site on voltage dependent anion channel 1. Both these processes provide enhanced energy supplies to the rapidly dividing cancer cells. In order to sustain such high requirements of mitochondrial metabolism a high supply of proteins and metabolites need to be supplied to mitochondria in addition to rapid increased exchange of ADP/ATP. Since the mitochondrial DNA consists of only 13 protein coding genes, majority of mitochondrial functional and structural proteins and metabolites are nucleus coded. These proteins/metabolites need to enter the mitochondria, a function that is performed by mitochondrial beta barrel protein TOM40 (translocase of mitochondrial outer membrane 40 kDa). Both TOM40 and VDAC1 are beta barrel proteins that are assembled from pre-proteins in mitochondria by the Sorting and Assembly Machinery 50 kDa (SAM50) protein. Cancer cells predominantly produce energy by glycolysis in cytoplasm rather than using ATP synthesis in mitochondria. It was hypothesized that change in the transport of proteins and metabolites into mitochondria due to downregulation of SAMM50 is responsible for this effect. Level of activity of SAMM50 can therefore be used as an indicator to determine the direction in which the breast cancer may be progressing leading to an early detection system.

BODY

While mitochondria have their own DNA, only 13 protein coding genes are expressed. The remainder of supply of proteins is nuclear in origin. These proteins include mitochondrial structural components as well as enzymes involved in respiration and mitochondrial metabolism. For all these molecules to reach their respective target sites in mitochondria, they have to be imported across the mitochondrial membranes – a function performed by the mitochondrial translocases. The main component of this setup is the translocase of outer mitochondrial membrane (TOM40). This beta barrel protein in association with other TOMs forms the main channel of transport across mitochondrial membrane. Another beta barrel protein called the SAM50 (sorting and the assembly machinery) is responsible for assembly and integration of TOMs in the outer

membrane. SAM is also responsible for the assembly of voltage dependent anion channel-1 (VDAC1) beta barrel protein, which the primary site for exchange of ATP/ADP and metabolites between mitochondria and cytoplasm. VDAC1 is also the primary site for binding of hexokinase, a major component involved in the Warburg effect responsible for conversion of hexose to hexose-6-phosphate. The role of mitochondrial beta barrel proteins and their synthesis machinery has not been reported with reference to tumor progression.

Of the three beta barrel proteins on mitochondrial outer membrane least or rather no information is available on the role of SAM50 in tumor development and progression. The information that is available on SAMM50 is indirect and not analyzed thoroughly. This information can be obtained from the NCBO Geo Profiles employing the search terms “SAM50 breast cancer”. From the profiles it has been observed that SAMM50 expression was more in cytoplasm (indicating RNA) than in the membrane fraction (8875697). In another study (49706118) the expression of SAMM50 was more in control set in comparison to treated set with miR-335; suggesting regulation of SAM50 pathway. In another study, hypoxia in breast cancer cells lead to induction of HIF1 and suppression of Sam50 expression (39950825).

TOM40 has been demonstrated to be a major protein found in plasma of patients suffering from pancreatic cancer (Ning et al., 2007).

Perhaps research efforts have gone more into the role and mechanism of VDACs in cancer cell regulation than the previous two beta barrel proteins. The bioenergetics of cancer cells is characterized by a high rate of aerobic glycolysis and suppression of mitochondrial metabolism (Warburg phenomenon). Mitochondrial metabolism requires inward and outward flux of hydrophilic metabolites, including ATP, ADP and respiratory substrates, through voltage dependent anion channels (VDAC) in the mitochondrial outer membrane. Although VDAC was once considered to be constitutively open, closure of VDAC is emerging as an adjustable limiter (governator) of mitochondrial metabolism. Studies of VDAC reconstituted into planar lipid bilayers show that tubulin at nanomolar concentrations decreases VDAC conductance. In tumor cell lines,

microtubule destabilizing agents increase cytoplasmic free tubulin and decrease mitochondrial membrane potential (ΔΨ), whereas microtubule stabilization increases ΔΨ. Tubulin-dependent suppression of ΔΨ is further potentiated by protein kinase A activation and glycogen synthase kinase-3β inhibition. Knockdown of different VDAC isoforms, especially of the least abundant isoform VDAC3, also decreases ΔΨ, cellular ATP and NADH/NAD+, suggesting that VDAC1 and VDAC2 are most inhibited by free tubulin. Tubulin-dependent closure of VDAC represents a new mechanism contributing to the suppression of mitochondrial metabolism in the Warburg phenomenon (Maldonado and Lemasters, 2012).

A major clinical problem regarding antitumoral treatment with DNA cross-linking agents such as cisplatin (Cisp), mechlorethamine (HN2) or its derivative melphalan (MLP) is intrinsic or acquired resistance to therapy, which frequently results from a resistance to apoptosis induction. Sharaf et al. (Biochemical Pharmacology, 2012) aimed to identify novel sensitizing targets to DNA cross-linker-induced cell death and demonstrated that MLP, Cisp and HN2 induce mitochondrial permeability transition pore (PTP)-mediated apoptosis in cervical and colon carcinoma cells. This apoptotic pathway is characterized by dissipation of the mitochondrial membrane potential, production of ROS, mitochondrial translocation of Bax, release of apoptogenic factors, caspase activation and nuclear alterations. The opening of PTP and subsequent apoptosis was reduced in Bax deficient cells and in cells with elevated Bcl-2 level, but not in cells invalidated for Bak. We further showed that, among the pro-apoptotic PTP regulators tested (VDAC1, creatine kinase, ANT1 and ANT3), exogenous overexpression of VDAC1 was the most effective in enhancing Cisp- and MLP-induced apoptosis. In addition, pharmacologically induced up-regulation of VDAC1 by the chemotherapeutic agent arsenic trioxide (As_2O_3) greatly sensitized HeLa cells to Cisp and MLP treatment. These data indicate that increased expression of VDAC1 appears as a promising strategy to improve DNA cross-linker-induced chemotherapy.

In line with the above ivestigation Brahimi-Horn et al (Cancer Research, 2012) identified the role of VDAC truncated form in imparting chemoresistance to tumor cells. They observed that mitochondria of certain hypoxic cells take on an enlarged appearance

with reorganized cristae. In these cells, it was found that voltage-dependent anion channel 1 (VDAC1), was linked to chemoresistance when in a truncated (VDAC1-ΔC) but active form. The formation of truncated VDAC1, which had a similar channel activity and voltage dependency as full-length, was hypoxia-inducible factor-1 (HIF-1)-dependent and could be inhibited in the presence of the tetracycline antibiotics doxycycline and minocycline, known inhibitors of metalloproteases. Its formation was also reversible upon cell reoxygenation and associated with cell survival through binding to the antiapoptotic protein hexokinase. Hypoxic cells containing VDAC1-ΔC were less sensitive to staurosporine- and etoposide-induced cell death, and silencing of VDAC1-ΔC or treatment with the tetracycline antibiotics restored sensitivity. Clinically, VDAC1-ΔC was detected in tumor tissues of patients with lung adenocarcinomas and was found more frequently in large and late-stage tumors. The findings demonstrate that via induction of VDAC1-ΔC, HIF-1 confers protection from apoptosis thereby maintaining ATP level and cell survival in hypoxia. Additionally, VDAC1-ΔC may hold promise as a biomarker for tumor progression in chemotherapy-resistant patients.

In a recent investigation Grills et al (PlosOne, 2011) identified VDAC1 as a marker for non-small cell lung cancer aggressiveness. The authors performed a meta-analysis on 602 individual expression profiles, to examine the impact of VDAC1 on survival. The results suggested that VDAC1 gene expression as a predictor of poor outcome in NSCLC and other cancers and are associated with dysregulation of a conserved set of biological pathways, which may be causally associated with aggressive tumor behavior.

Key Research Accomplishments

In order to assess the role of SAM50, 40 and VDAC1 in tumor cell growth, cultured breast cancer cell lines of variable genetic backgrounds were taken. Cells were procured from ATCC. Table 1 delineates the cell lines employed in the study and their genetic characteristics.

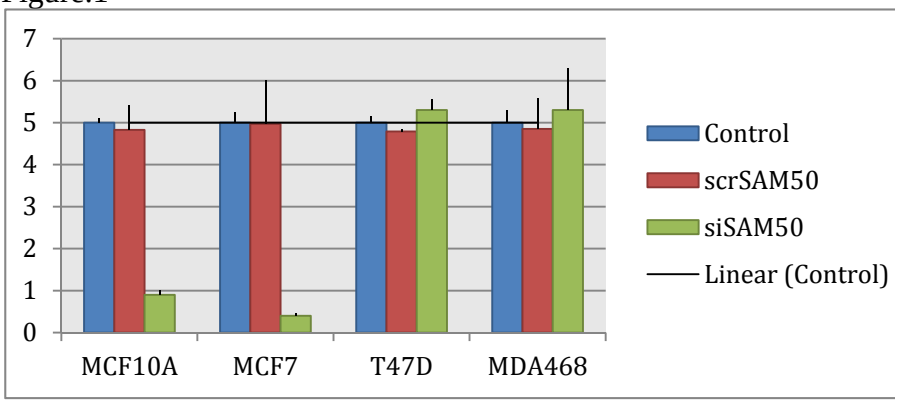
Table 1. Characteristics of breast cancer cell lines

Cell line	ER	PR	ERBB2/HER2	TP53 mutation	Tumor Type
MCF 10A	-ve	-ve	-ve	WT	Tumorless
MCF 7	+ve	+ve	-ve	WT	Metastatic adenocarcinoma
T47D	+ve	+ve	-ve	Mutant	Invasive Ductal Carcinoma
MDA468	-ve	-ve	-ve	Mutant	Metastatic adenocarcinoma

On the abovementioned backgrounds, the siRNA against SAM50, TOM40 and VDAC were transfected independently in all the cell lines. Lipofectamine 2000 (Sigma) was used as the transfection agent following the manufacturers protocol. Additionally, scrambled siRNA for each of the genes was used as control. Following the siRNA treatment, cell proliferation was estimated by counting the number of cells using the Trypan Blue assay in a TC-10 cell counter (BioRad). To understand the modulation in the cell development after siRNA to different mitochondrial proteins the following genes were analyzed by real-time PCR and western blotting – Sam50, TOM40, VDAC1, COXIV, Cyclin D1, mTOR, SNAI1, SNAI2, Vimentin and ZEB1.

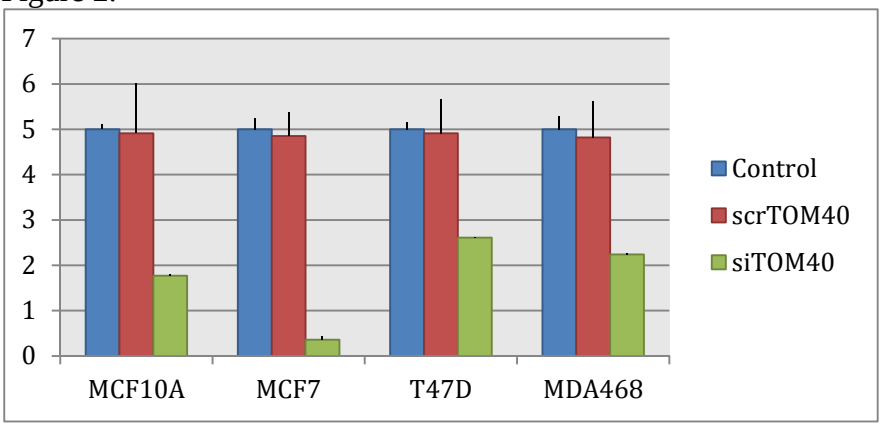
The cell proliferation (measured by cell counter) was variable in each of the siRNA treatments. After treatment siSAM50, the MCF10A and MCF7 cell proliferation was suppressed by about 90%. The results for T47D and MDA468 were surprisingly opposite and did not demonstrate a suppression of proliferation (Figure 1). It is noteworthy that MCF7 had almost double the suppression in proliferation than the normal cell line MCF10A. The cell count on the vertical axis is x10^3 cells.

Figure.1



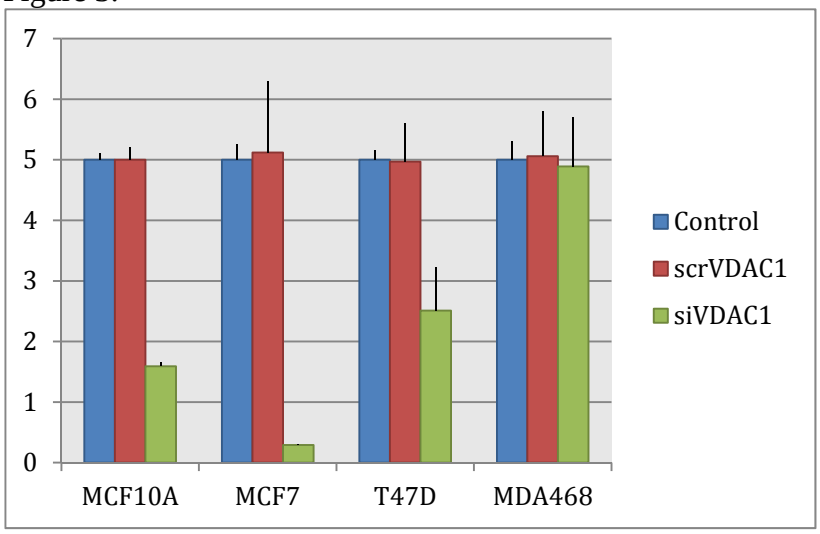
The siTOM40 results demonstrated that irrespective of the genetic background of the tumor cell line there was suppression in cell proliferation (Figure 2.). Although the level of suppression was more in MCF10A and MCF7 cells, the T47D and MDA468 had relatively less suppression than other cell lines. The cell count on the vertical axis is $x10^3$ cells.

Figure 2.



The variability in the siVDAC treated cell proliferation demonstrated that MCF10A, MCF7 and T47D proliferation was suppressed. On the other hand there was no significant variation in the cell counts of the MDA468 cell line (Figure 3.). The cell count on the vertical axis is $x10^3$ cells.

Figure 3.

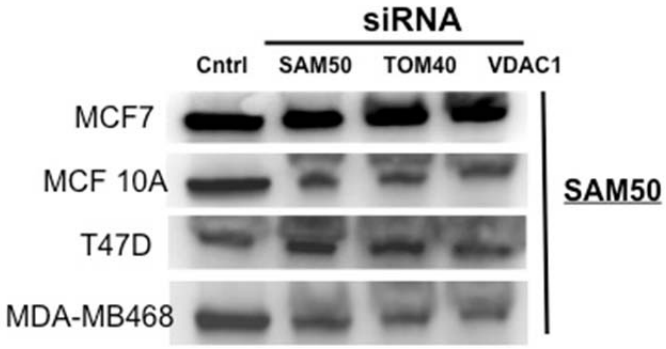


Following the cell proliferation assay the cells were analyzed for key proteins responsible for various cellular mechanisms, e.g. SAM50, TOM40, VDAC1 and COXIV

for mitochondrial function, mTOR for protein synthesis, Zeb1 for transcriptional machinery activation leading to EMT/metastasis, SNAI1 (Snail) and SNAI2 (Slug) for metastatic potential, Cyclin D1 and Vimentin for cell division and proliferation.

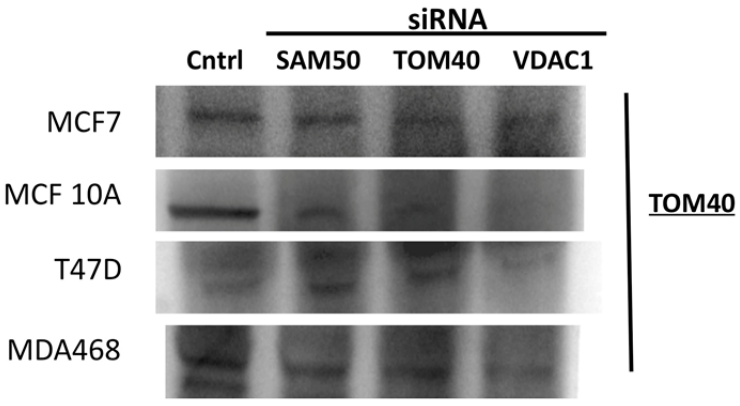
While SAM50 knockdown with siRNA did suppress cell proliferation, the amount of protein in the cell did not show much variation in the MCF7 cell line when analyzed by western blotting (Figure 4). On the other hand all the other cell line did demonstrate a reduction in the amount of SAM50 protein, although only marginally. Also, the levels of SAM50 were increased in T47D cell line under both siSAM50 and siTOM40 conditions. The levels of SAM50 were significantly suppressed when VDAC1 was knocked down in MDA468 cell line.

Figure 4



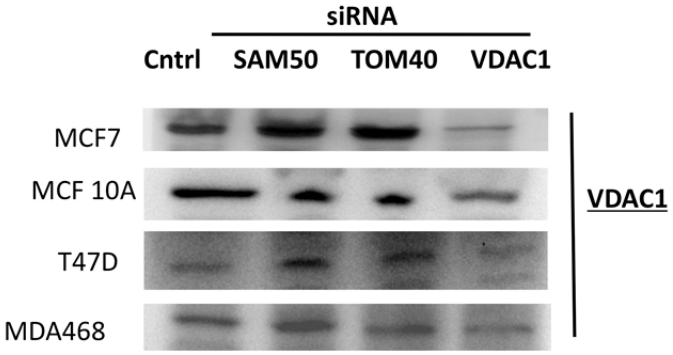
After the TOM40 knockdown, the levels of TOM40 in the different cell lines showed variation. Maximum variation in TOM40 levels was observed in MCF10A and T47D (Figure 5.). MCF7 did not demonstrate significant variation from control in TOM40 levels under either of beta barell knockdowns. Contrary to this, TOM40 level was almost completely abolished when VDAC1 was knocked down in T47D. T47D also had lower amounts of TOM40 under non-treated condition.

Figure 5.



VDAC1 levels under different treatments showed significant deviation from non-treated control. VDAC was reduced about 90% in MCF7, while it was increased under siSAM50 and siTOM40 (Figure 6.). Interestingly, the level of VDAC1 protein did not reduce as much in MCF10A as it did in other cell lines. Even under siSAM50 and siTOM40 treatment VDAC1 level was similar to that of control. This was also the case with MD468. This probably could be due to the fact that VDAC is one of the most abundant mitochondrial protein with a very high turnover rate.

Figure 6.



Under different mitochondrial beta barrel knockdown conditions respiratory chain enzyme COXIV was increased in MCF7 lines (Figure 7.). While siSAM50 increased the amount of COXIV, siTOM40 significantly reduced the levels of COXIV, probably due to reduction in the transfer of COX pre-proteins. Similar result was observed in MD468. In T47D cell line also siSAM50 resulted in increase of COXIV in comparison to control, though siVDAC reduced it below control level. In MCF10A while siSAM50 increased the COXIV level, siTOM40 did not result in a significant variation to that of control. These observations suggest that there is variability in the requirement of COXIV in different cell lines, since all the cell lines demonstrated similar proliferation patterns in trypan blue assay.

Figure 7.

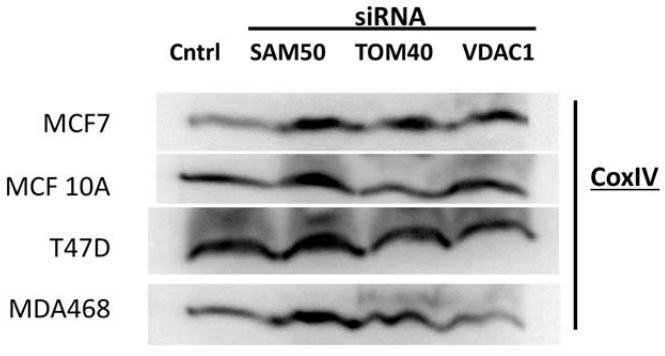
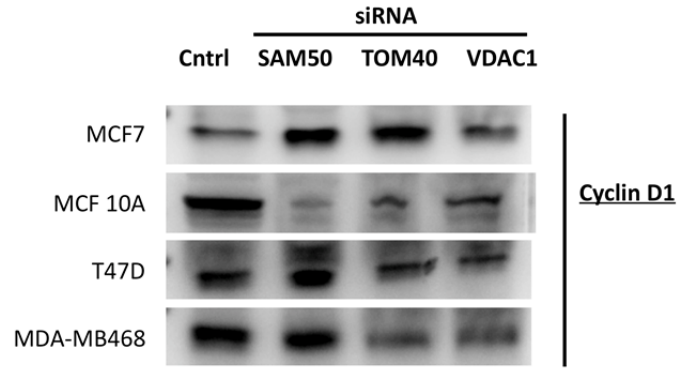


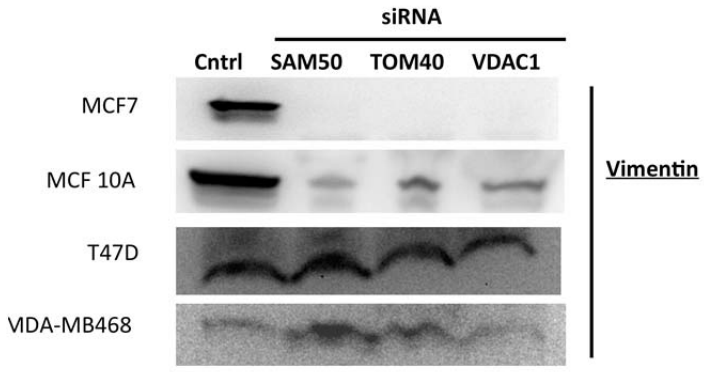
Figure 8.



Cyclin D1 and Vimentin antibodies were employed to assess the variation in cell cycle driving proteins under various mitochondrial protein knockdown condition. As has been the case with COXIV, cyclin D1 in MCF7 and T47D demonstrated an increase with respect to control. On the other hand, in MCF10A cyclin D1 was almost depleted under siSAM50 and significantly reduced under siTOM40 and siVDAC1 (Figure 8.). In MDA468, while siSAM50 did not reduce the amount of cyclin D1, siTOM40 and siVDAC did suppress the cyclin D1 levels.

Vimentin levels were completely depleted in MCF7 in either of the mitochondrial protein knockdown condition (Figure 9.). MCF10A also demonstrated lower levels of vimentin under knockdown conditions. Contrary to this T47D did not show any variation in the vimentin levels across the panel. MDA468 on the other hand showed an increase with respect to control under siSAM50 and siTOM40 conditions. The level of vimentin under siVDAC1 condition was similar to that of control for MDA468 cells.

Figure 9.



To assess the effect of siRNA treatment against the three mitochondrial beta barrel proteins on the metastatic mediators in the mentioned cell lines antibodies against

SNAI1 (Snail) and SNAI2 (Slug) were employed. MCF7 showed a slight reduction in Slug in MCF7, MDA468 and T47D cell lines (Figure 10). The levels of Slug were similar to those of control cells in MCF10A. On the other hand Snail was completely abolished in MCF7. In contrast it expression was enhanced in MCF10A and MDA468. The level of Snail remained unchanged in T47D cell line (Figure 11).

Figure 10.

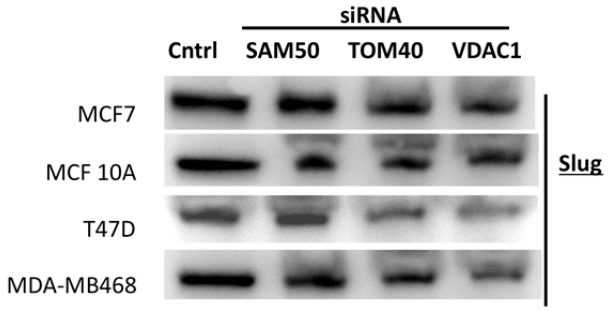
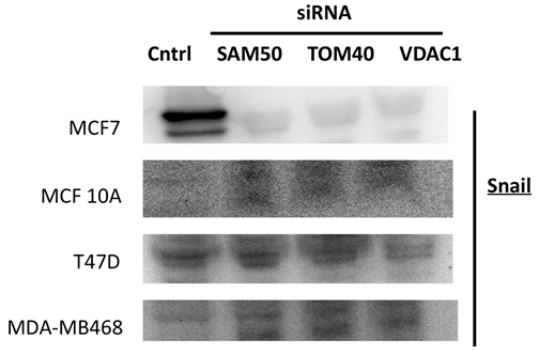
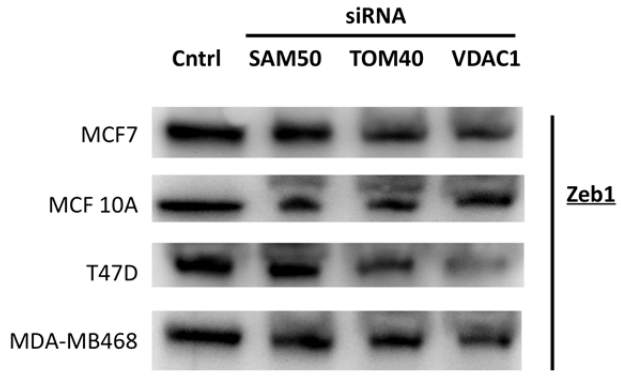


Figure 11.



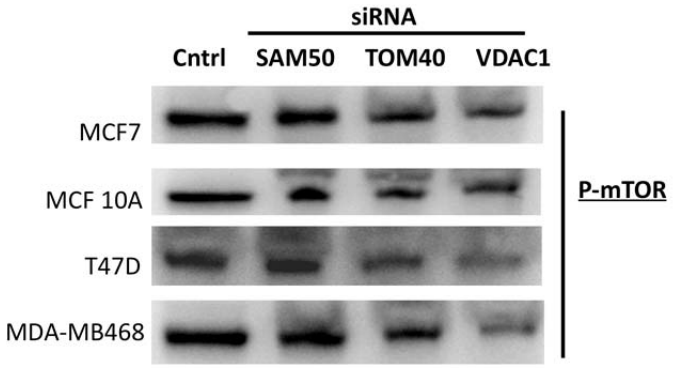
To assess the impact of mitochondrial protein knockdown on the transcriptional regulation of metastatic markers, Zeb1 levels were assessed in all the cell lines after knockdown. It was observed that siSAM50 did not result in reduction in Zeb1 levels in any of the cells tested. This reduction was more evident in siTOM40 and siVDAC1 in MCF7 and T47D with more being in T47D.

Figure 12.



mTOR levels were analyzed after knockdown treatment to gain an insight into the protein stimulatory machinery. To do this phospho-mTOR antibody was employed. It was observed that there was a marginal reduction in phosphorylated mTOR under siTOM40 and siVDAC1. This suppression was more pronounced in T47D cell line. And under siVDAC1 in MDA468 cells.

Figure 13.



Reportable Outcomes

The following observations were made after the investigation:

1. MCF7 cells are more resistant to SAM50 and TOM40 knockdown. This could be due to their ability to activate the transcription machinery to compensate for the loss due siRNA.
2. All the cell lines try to compensate for the loss of mitochondrial beta barrel proteins by upregulating the respiratory enzyme COXIV.
3. While suppression of SAM50, TOM40 and VDAC1 could result in suppression of epithelial-mesenchymal transition in MCF7, it would prove counter productive in MDA468, where the expression of Snail was enhanced after knockdown.
4. Since TOM40 is the main channel of entry of nuclear encoded proteins, its knockdown should result in suppression of mitochondrial activity. The results of western blot suggest contrary to this in some cases. This could be a result of negative feedback loops originating from mitochondria, but this hypothesis needs to be investigated in detail.

5. The variation in the level of vimentin between different cell lines suggests that while progression of some metastatic adenocarcinomas like those similar to MCF7 type may be treated with mitochondrial protein knockdown, this will result in promoting aggressiveness in other categories of breast tumors.

Conclusion

It is evident from this preliminary data that mitochondrial beta barrel proteins play a crucial role in progression and development of tumorigensis in breast cancers. It is important to realize that knocking down of either of the proteins in every class of tumor will not result in a similar outcome. In some cases it may enhance tumorigenesis while in other cases it may yield suitable results in curtailing carcinogenesis. Unlike the response of mentors in this study, detailed investigations by scientists who are interested in pursuing this study should be undertaken.

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